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The protective effect of icariin in testosterone-induced benign prostatic hyperplasia in rats via suppression of macrophage infiltration

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ABSTRACT

Benign prostatic hyperplasia (BPH) is a disorder that affects males at the age of 40. Icariin (ICA) is a flavonoid reported to treat impotence, atherosclerosis, and osteoporosis. This study aimed to investigate the potential protective effect of ICA in rats with testosterone-induced BPH. ICA prevented increased prostate weight and prostate index levels compared to animals with BPH. Histological examination showed that ICA significantly ameliorated histological changes in the prostate tissues compared to the BPH group. In addition, ICA significantly improved testosterone-induced oxidative stress evidenced by lowering MDA levels, as well as enhancing the levels of antioxidants including GSH, CAT, GPx and SOD. Moreover, compared to the BPH group, ICA prevented the accumulation of collagen fibres and the expression of α-smooth muscle actin (α-SMA). ICA was also able to prevent testosterone-increased macrophage accumulation in prostate tissues evidence by reducing the expression of F4/80 marker. In conclusion, this study provides a new evidence that ICA experimentally attenuates testosterone-induced BPH in rats, partially by inhibiting macrophage accumulation.

Keywords: Icariin, BPH, macrophages

1. INTRODUCTION

Benign prostatic hyperplasia (BPH) is described as uncontrolled proliferation of prostatic glandular epithelial and stromal cells that inflict a non-malignant enlargement of the prostate gland. The disease begins in men at the age of 40, and the incidence increases with age to affect 50% and 90% of men at 60 and 80 years old, respectively (Lepor, 2005; Roehrborn, 2008). Androgens stimulate several growth factors leading to prostatic overgrowth (Corona et al., 2014; Izumi et al., 2013). Testosterone, the main male sex hormone, is converted in prostatic cells by 5α–reductase to a powerful androgen dihydrotestosterone (DHT) that plays a key role in BPH progression (Wen et al., 2015; Izumi et al., 2013). Patients with BPH suffer from lower urinary tract symptoms (LUTS),

which significantly affect their quality of life. LUTS can be classified into irritative symptoms (including increased frequency and urgency of urination and nocturia) or obstructive symptoms (such as incomplete voiding, hesitancy, and incontinence) (Gacci et al., 2012; Bauman et al., 2014; Ma et al., 2012; Patel and Parsons, 2014). Apart from androgens, other factors that contribute to hyperplasia include oxidative stress, inflammation, and reduction of stromal apoptosis (Madersbacher et al., 2019; Corona et al., 2014). The cornerstone therapeutic agents for BPH are α1A antagonists and 5α–reductase inhibitors. However, with prolonged use, both drug categories can increase the risk of fibrosis and collagen disposition (Bauman et al., 2014; Ma et al., 2012).

Natural products have emerged as important sources to alleviate BPH in different animal models (Li et al., 2018). These phytotherapies have many benefits, including anti-proliferative, antioxidant, anti-inflammatory and anti-fibrotic properties and inhibition of collagen deposition (Liu et al., 2018; Deng et al., 2019; Park et al., 2018). Icariin (ICA) is a flavonoid isolated from herbaceous flowering plants belonging to Epimedium (family Berberidaceae). ICA is reported to treat conditions like impotence, atherosclerosis, and osteoporosis (Fang and Zhang, 2017; Ye and Chen, 2001; Liu et al., 2005). In addition, several works have shown that ICA exhibits an anti-proliferative effect on several cancer cell lines, including prostate cancer cells. This anticancer effect is mainly undertaken through an apoptosis-inducing effect and immunomodulation (Tan et al., 2016). Whether ICA can exhibit a protective effect against BPH remains unclear. Therefore, our study aimed to examine the effect of ICA on testosterone-induced BPH in rats by investigating (1) the histological changes in prostate tissues, (2) oxidative stress status and antioxidant activities, (3) collagen disposition, and (4) macrophage infiltration.

2. MATERIALS AND METHODS

Reagents and Chemicals

Testosterone enanthate (Testone®) was obtained from Chemical Industries Development Co. (CID), Giza, Egypt). Icarrin with purity of more than 98% was obtained from Liftmode, Synaptent LLC (Chicago, USA).Chemicals were of analytical quality. Antibodies against α-smooth muscle actin (α-SMA; ab5694) from Abcam (Cambridge, Uk). Anti-F4/80 (sc-26643-R) was purchased from Santa Cruz Biotechnology, Dallas, USA).

Animals

Male Wistar rats (n=32, 10 weeks old, weight 220±30g) from King Fahd Medical Research Centre, King Abdulaziz University were kept in a ventilated room on 12 h light/dark cycle and with standard rodent diet and water. A week prior to the experiment, the rats were adapted to the above conditions for one week before starting the study. The protocol was approved by The Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (reference number 1438-107).

Study design

Animals were divided into four groups in a random manner (n=8/group): i) Control group (C)was given 0.3 ml/kg/day of dimethylsulfoxide (DMSO)(p.o.), the vehicle of ICA, and 1 ml/kg/dayof olive oil (s.c.), the vehicle of testosterone; ii) BPH model group (BPH); iii) BPH + ICA(25 mg/kg)group; and iv) BPH+ ICA(50 mg/kg) group. To induce BPH, testosterone (5 mg/kg/day) was administered subcutaneously to all groups, with the exception of control group, as previously described (Abdel-Naim et al., 2018). The third and fourth groups were treated daily with ICA (i.p) at the stated doses. The protocol was continued for 14 consecutive days. At the conclusion of the study, the rats' weights were recorded and the rats were euthanized to dissect the prostates. Both ventral and dorsal lobes of the prostate were collected and held in formalin (10%) for histology and immunohistochemistry. Remnant prostate tissues were kept at −80°C until further analysis.

Prostate Indices and Weights

Determination of the prostate index (PI, mg/g) for each rat was as such: Prostatic index = (prostate weight (mg)/body weight (g))*1000.

Histopathology

After fixation of prostate tissues in formalin, they were paraffinized and sectioned (5 μm thickness). Hematoxylin-eosin (H&E) or Masson's Trichrome were used for staining the fixed sections. A minimum of 3 slides were analyzed and photographs were obtained using a light microscope (Nikon Eclipse TE2000-U, NIKON, Japan).

Immunohistochemistry

The immunohistochemical staining of F4/80 andα-SMA was performed in prostate tissues as previously described (Neamatallah et al., 2019). Briefly, prostate sections were deparaffinized, rehydrated, and placed in citrate buffer (pH 6) for 25 min. 3% (v/v) H_2O_2 in methanol was used for 25 minat ambient temperatures to block peroxidases. Non-specific reactions were carried outfor 25 min with normal goat serum, 10% (v/v) in phosphate buffered saline and Tween 20 (PBST). Incubation with primary antibodies F4/80 andα-SMA was followed. This was followed by washing the sections using PBST and a 1 hour incubation in peroxidase conjugated secondary antibody in 3% Bovine serum albumin (BSA/PBS). 10% (v/v) 3, 3-diaminobezidine (chromogen substrate) in stable hydrogen peroxide was used for visualization of the immune-stained slides. Images were captured with a light microscope (Nikon Eclipse TE2000-U, NIKON, Japan). At least three sections per rat were photographed and quantified using software (Image J, 1.46a, NIH, USA).

Determination of oxidative stressmarkers and antioxidants

Prostate tissue homogenization was carried out in ice cooled phosphate buffered saline + 1% Triton x100, pH 7.4. Prostatic oxidative stress markers and antioxidants including malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GPx) as well as superoxide dismutase (SOD) and catalase (CAT) were assessed using commercially available kits (Biodiagnostics, Giza, Egypt). Results were normalised to prostate weight.

Statistical Evaluation

Results are presented as mean ± SD. To assess statistical significance, one-way ANOVA followed by Tukey as a post hoc test was used between examined groups. All analyses were carried out using GraphPad Prism 6 and differences were taken as significant at $p < 0\ 05$.

3. RESULTS

Effect of ICA on prostate growth in BPH rats

The administration of ICA at both doses was generally tolerated and no mortality or abnormal clinical observations were detected inthe treated animals during the study. In addition, both doses of ICA did not significantly affectrats' body weight. However, prostate weight and PI significantly raised by 3 and 2-folds, respectively, in comparison with that of control rats ($p < 0.05$). Treatment with either 25 or 50 mg/kg of ICA caused a significant lowering in the prostate weight and PI relative to the BPH group ($p < 0.05$) (Table 1).

Table 1 Effect of ICA on prostate growth

Group	Final rat weight (g)	Prostate weight (mg)	PI
Control	238.66 ±32.38	0.6 ± 0.072	2.74 ± 0.44
BPH	320.17 ± 19.51	1.78 ± 0.25	5.55 ± 0.72[a]
BPH + ICA 25 mg/ml	290.5 ± 31.85	1.35 ± 0.09	4.45 ± 0.19[b]
BPH + ICA 50 mg/ml	308.67 ± 40.13	1.13 ± 0.2	3.9 ± 0.78[b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. BPH group

Effect of ICA on oxidative stress markers

The effect of ICA on modulating prostatic oxidative stress generated by testosterone treatment was evaluated by determining levels of MDA, GSH, SOD, GPx and CAT. MDA concentrations as a marker for lipid peroxidation, was notably elevated in the BPH group compared to controls ($p < 0.05$).

Table 2 Effect of ICA on oxidative stress markers

Group	MDA (nmol/g)	GSH (mg/g)	CAT (U/g)	GPx (U/g)	SOD (U/g)
Control	9.94±17.35	21.85±4.33	0.61±0.07	24.56±0.93	657.57±64.66
BPH	23.01±4.91[a]	13.2±1.86[a]	0.13±0.01[a]	6.81±3.73[a]	321.55±66.74[a]
BPH + ICA 25 mg/kg	20.04±1.99[a,b]	10.06±1.76 [a,b]	0.33±0.16 [a,b]	7.93±5.03 [a,b]	396.58±77.58 [a,b]
BPH + ICA 50 mg/kg	12.32±0.98[b]	21.40±1.86[b]	0.6±0.05[b]	19.1±3.56 [a,b]	557.28±36.06[b]

[a]P<0.05 vs. control group; [b]P<0.05 vs. BPH group

Testosterone also lowered the prostate glands' total antioxidant capacity as shown by the significantly reduced the levelsof GSHas well as CAT, GPx, and SOD activities ($p < 0.05$). Although the 25 mg/kg dose of icariin partially ameliorated these oxidative stress markers relative to the testosterone-treated group, intraperitoneal administration of ICA (50 mg/kg) significantly restored levels of GSH, CAT, GPx and SOD relative to the BPH group ($p < 0.05$) returning them to similar levels as the control animals. MDA levels in ICA-treated group were reduced significantly ($p < 0.05$) relative to the BPH group (Table 2) and were comparable with controls.

Effect of ICA on prostatic structure in BPH rats

The histological analysis of prostate dorsal and ventral lobes of control rats showed normal morphological structure as prostatic acini were in normal size and shape lined by cuboidal epithelium showing regular degree of folding and surrounded by a thin fibromuscular stroma (Figures 1A and E). In contrast, sections of BPH rats showed disruption of prostatic morphology in which the thickness of epithelial layer was obviously increased relative to the control group (Figures 1 B, F and I). Prostatic acini were in irregular shape with marked hyperplasia, narrow lumen, and profound intra-luminar projections. The Inter-acinar stromal tissue showed numerous dilated bloods vessels with increased accumulation of fine collagen fibers and inflammatory cells (Figure 1 B and F). ICA treatment at 25 and 50 mg/kg ameliorated the changes in prostate histological appearance. It decreased epithelial hyperplasia and intraluminal fronds in a dose dependent manner. In addition, ICA-treated groups showed gradual restoration in acinar shape and size, in which the luminal spaces were distended and filled with reticulated secretions (Figures 1C, D, G and H).

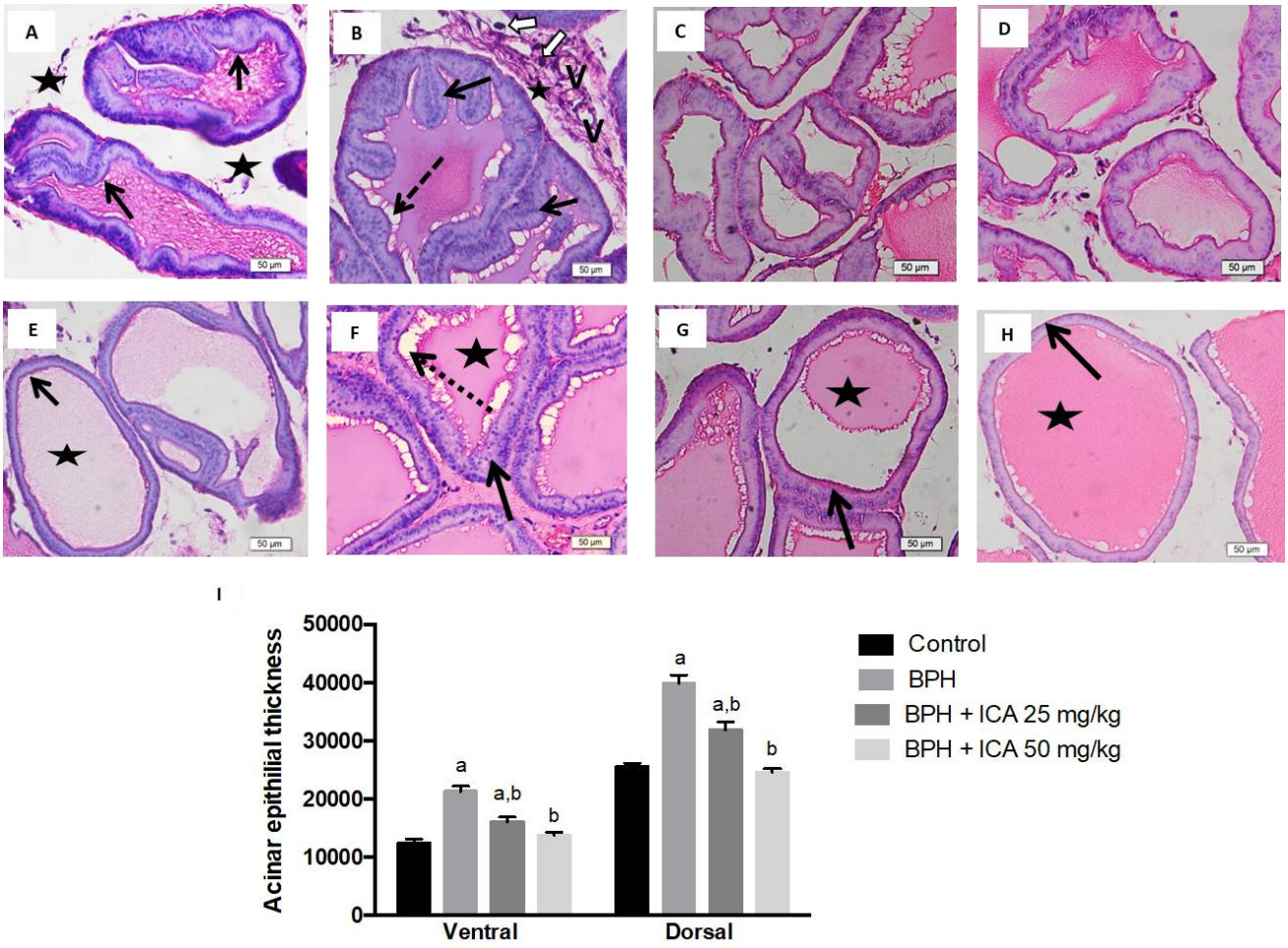

Figure 1 Sections from rats' dorsal prostate (A-D) and ventral prostate (E-H) stained by H&E. (A,E) Control rat showing normal prostate as prostatic acini are lined by cuboidal epithelium with relative degree of folding (black arrows). The lumen contains reticulated secretion and inter-acinar stroma is scanty showing few fibers and cells (stars). (B, F) Sections from BPH rats showing adenomatous hyperplasia with an increase in the height of lining epithelium (black arrows). The luminal secretion is dark-stained showing peripheral vacuoles separating it from the epithelial surface (dotted arrows). The inter-acinar stroma showed numerous dilated small blood vessels (V) with increased the deposition of fine collagen fibers (black star) and infiltration of immune cells (white arrows). (C, G) Sections from testosterone + ICA (25 mg/kg) rats showing slight normalization of prostate structure. (D, H) Sections from testosterone + ICA (50 mg/kg) rats showing clear reduction in hyperplasia. (I) measurements of acinar epithelial thickness in the studied groups expressed as mean ± S.E.M. (n = 6). $^{a}P < 0.05$ vs. control group; $^{b}P < 0.05$ vs. BPH group.

Effect of ICA on collagen disposition and α-SMA expression in BPH rats

Few collagen fibers in the inter-acinar stroma (Figures 2 A and E) were seen in the Masson's trichrome-stained sections of the dorsal and ventral lobes of the prostate glands of control rats. However, the prostatic sections from BPH model showed apparent accumulation of collagen in comparison to controls (Figures 2 B and F). ICA-treated rats at 25 and 50 mg/kg showed minor prostatic collagen deposition (Figures 2 C, D, G and H). Furthermore, ICA administration affected the expression of smooth muscle fibres marker (α-SMA). As presented in figures 3A, B, E and F, BPH rats showed significant positive α-SMA immune-reactive smooth muscle fibers surrounding the acini when relative to controls. There was a dose-dependent reduction in α-SMA expression observed in ICA treated rats compared to untreated BPH animals (Figures 3C, D, G and H).

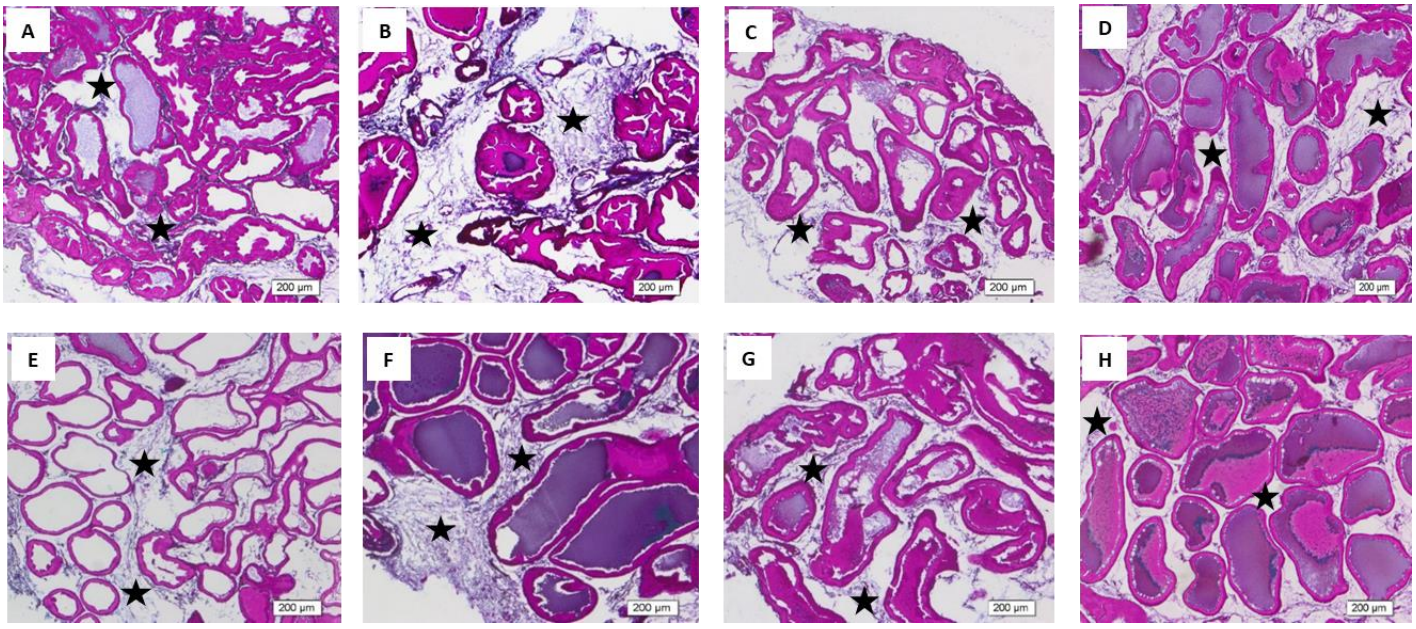

Figure 2 Sections from rats' dorsal prostate (A-D) and ventral prostate (E-H) stainedby Masson's trichrome for collagen disposition (stars). (A, E) Sections from control rats. (B, F) Sections from BPH rats showing marked deposition of collagen fibers between acini (interstitial fibrosis). (C, G) Sections from testosterone + ICA (25 mg/kg) rats showing decreased degree of fibrosis. (D, H) Sections from testosterone + ICA (50 mg/kg) rats showing few fine collagen fibers similar to the control group.

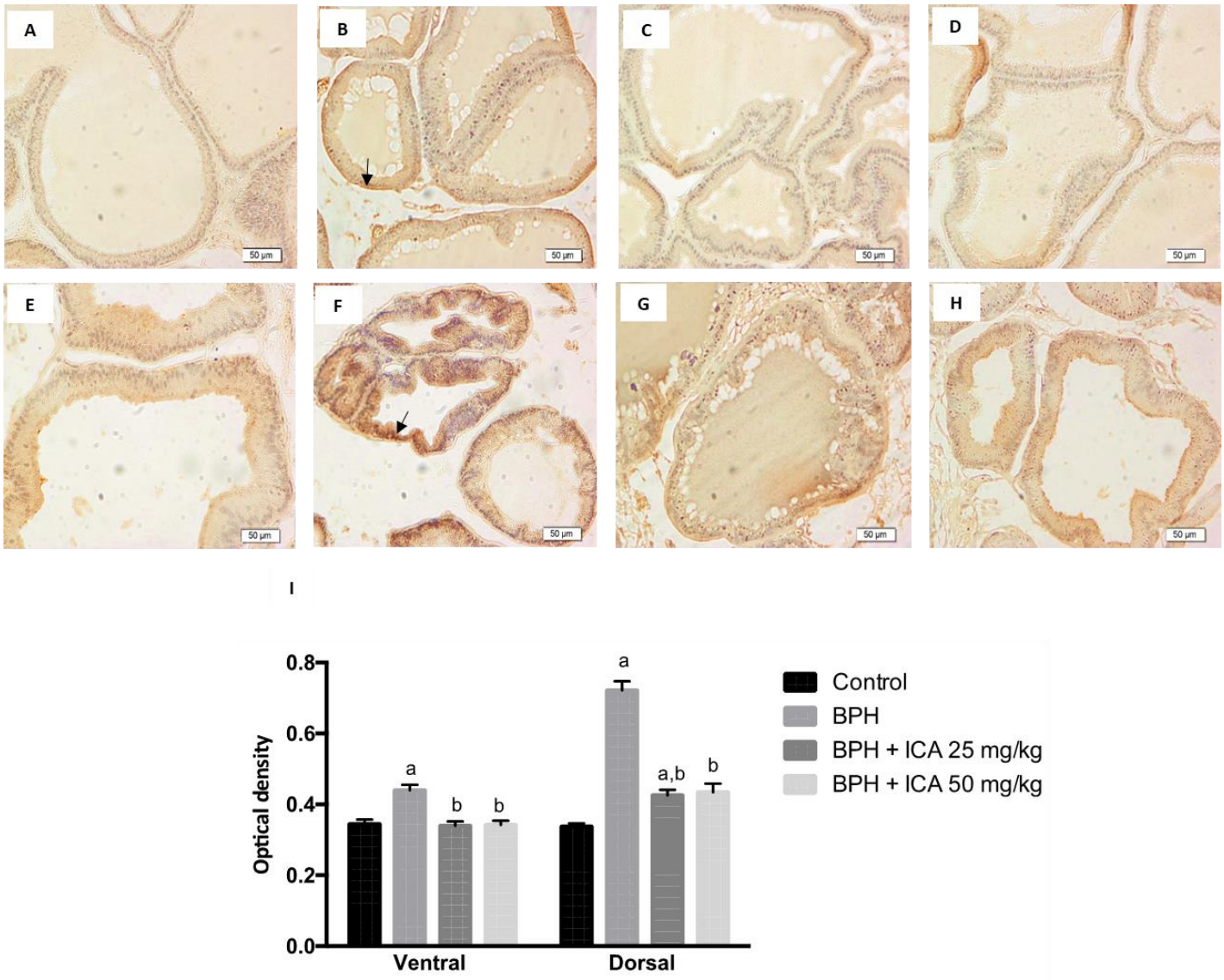

Figure 3 Sections from rats' dorsal prostate (A-D) and ventral prostate (E-H) immune-stained for α-SMA. (A, E) Sections from control rats. (B, F) Sections from BPH rats showing excessive expression of α- SMA. C: (C, G) Sections from testosterone + ICA (25 mg/kg) rats showing mild expression of α- SMA. (D, H) Sections from testosterone + ICA (50 mg/kg) rats showing expression of α- SMA resembles to that of the control group. (I) α- SMA expression quantification by the Image J analysis system expressed as mean ± S.E.M. (n = 6). $^{a}P < 0.05$ vs. control group; $^{b}P < 0.05$ vs. BPH group.

Effects of ICA on macrophage infiltration in BPH rats

Figures 4 show the role of ICA on the modulation of prostatic F4/80 expression of F4/80, a common macrophage marker. The rats from BPH group showed higher expression of F4/80relative to controls. On the other hand, administering ICA caused a marked reduction of F4/80-positive cells in the prostate of treated mice compared to BPH rats. This suggests a significant reduction of macrophage population, when BPH rats were treated with ICA.

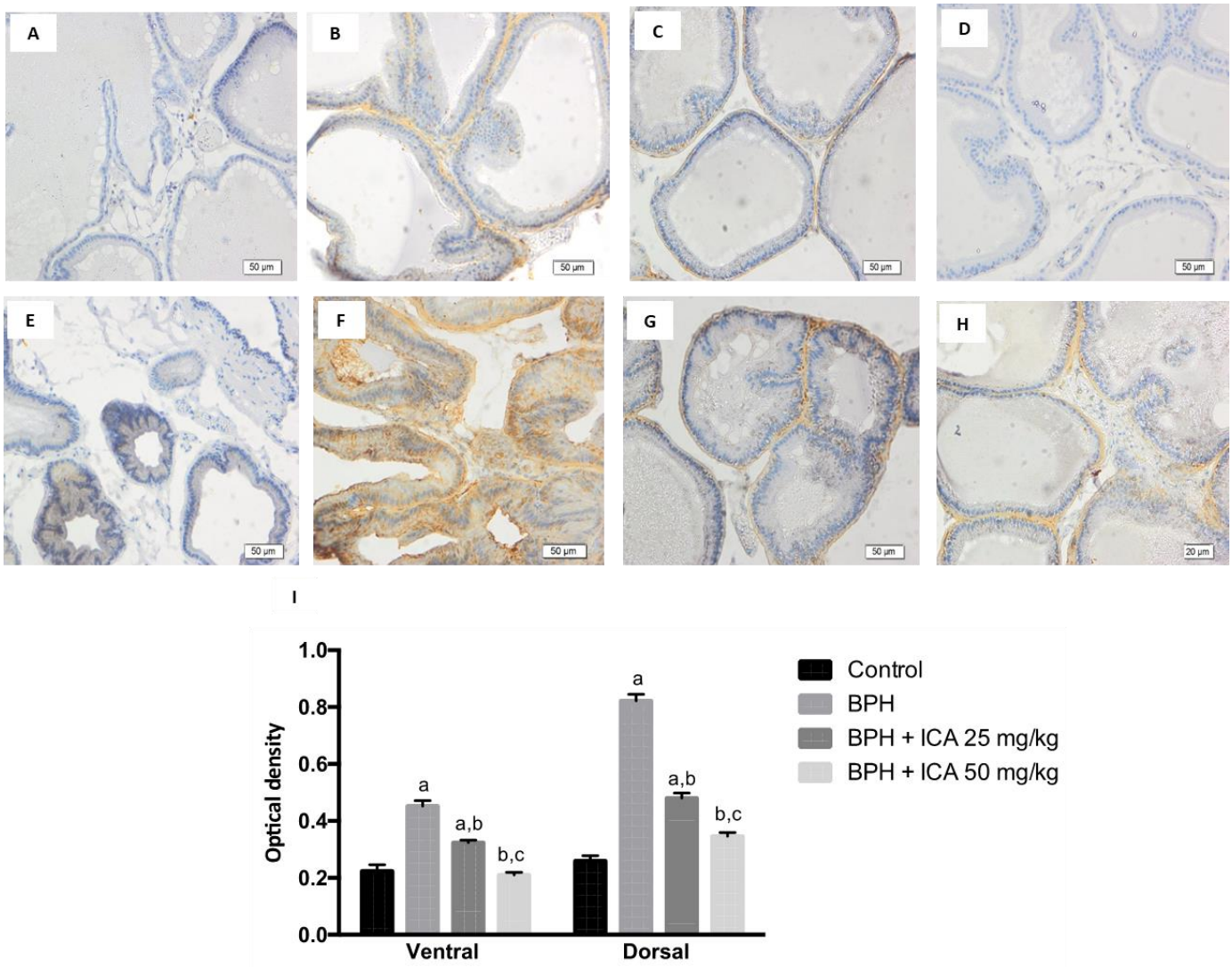

Figure 4 Sections from rats' dorsal prostate (A-D) and ventral prostate (E-H) immune-stained for F4/80. (A, E) Sections from control rats. (B, F) Sections from BPH rats showing excessive expression of F4/80. (C, G) Sections from testosterone + ICA (25 mg/kg) rats showing mild expression of F4/80. (D, H) Sections from testosterone + ICA (50 mg/kg) rats showing expression of F4/80resembles to that of the control group. (I) F4/80 expression quantification by the ImageJ analysis system expressed as mean ± S.E.M. (n = 6). $^{a}P < 0.05$ vs. control group; $^{b}P < 0.05$ vs. BPH group; $^{b}P < 0.05$ vs. ICA (25 mg/kg) group.

4. DISCUSSION

BPH is regulated by the production of androgens, which increase with age to promote proliferation and inhibit prostate glandular epithelium cell death (Shin et al., 2012; Izumi et al., 2013). Testosterone (or its metabolite, DHT) can bind to androgenic receptors (ARs) to trigger several growth factors, such as TGF-β, bFGF and VEGF, which further promote the growth of the prostate (Timms and Hofkamp, 2011; Lei et al., 2014a; Lei et al., 2014b). In the current study, testosterone was used to experimentally develop BPH in male rats. This model, based on subcutaneous administration of testosterone, was successfully utilized in several other studies (Al-Trad et al., 2019; Wu et al., 2017; Abdel-Aziz et al., 2020; Abdel-Naim et al., 2018). The aim was to study ICA's capability to attenuate testosterone-induced BPH in rats. We showed that testosterone at 5mg/kg/d was able to induce glandular enlargement and hyperplastic prostatic changes. This was evidenced by raised prostate weights and indices as well as altered histopathological architecture. The treatment of animals with ICA for two weeks prevented testosterone-induced increases in prostate weight and index, with 50 mg/kg showing significantly better effect than 25mg/kg. A marked improvement in prostatic acini histological features was noted in the ICA-treated group. In addition ICA attenuated glandular epithelial height, hyperplasia and intraluminal papillary projections. This could be attributed to the anti-proliferative effect of ICA. Specifically, ICA inhibited prostate cancer cells growth by arresting the G1 cell cycle phase (Huang et al., 2007). Furthermore, ICA was able to stop the growth and migration of

human prostate epithelial cell line RWPE-1 and benign prostatic hyperplasia epithelial cell line BPH-1. ICA also activated the intrinsic pathway of apoptosis in several other cancer cell lines (Li et al., 2013; Li et al., 2014; Sun et al., 2015; Wu et al., 2015). These initial findings propose ICA as a promising agent to control BPH, suggesting its anti-proliferative action against prostatic epithelial cells as a primary target.

In the present investigation, testosterone treatment increased the disposition of collagen fibres, as evidenced by Masson's trichrome stain. Other previous work reported a similar effect in rat models of BPH induced by testosterone (Morcos and Afifi, 2011; Wu et al., 2017). Here, ICA treatment caused a major decrease in the content of collagen in prostatic lobes. It has been established that several growth factors triggered by testosterone play critical roles in regulating stromal cell proliferation and differentiation as well as collagen accumulation during BPH development. The stromal mass in BPH contains mainly collagen fibres and smooth muscle cells (Rosenzweig-Bublil and Abramovici, 2006). In particular, it was suggested that increased collagen content resulted from the profibrotic TGF-β1 that was upregulated in the rats' prostates with BPH (Mauviel, 2005; Al-Trad et al., 2017). The present work also showed that ICA decreased testosterone-enhanced expression of α-SMA, a defined marker for the transformation of fibroblasts into myofibroblasts. This differentiation process is induced by TGF-β1, and both cells are involved in the synthesis of stromal fibrillary constituents and eventually lead to prostatic fibrosis in prostatic hyperplasia (Hata et al., 2020). Thus, our findings strongly suggest that ICA attenuated BPH in rats though inhibiting collagen disposition and fibrosis.

Several studies have implicated oxidative stress in the development of testosterone-induced BPH (Ren et al., 2015; Atawia et al., 2013; Vital et al., 2016). Consistently, we showed that testosterone induced oxidative stress in prostate tissues, as it increased MDA levels and decreased antioxidant enzyme activities including CAT, GPx, SOD and non-enzymatic GSH. However, ICA displayed noticable antioxidant properties, as it reduced the raised in MDA and enhanced antioxidant activities similar to the control group. This is consistent with the well-documented antioxidant properties of ICA and its protection against several conditions (Jia et al., 2019; Xiong et al., 2014; He et al., 2020). ICA holds a structure of 8-prenyl flavonoid glycosides that can stabilize reactive oxygen species to afford strong antioxidant effects. Therefore, our data suggest that ICA attenuates oxidative stress following testosterone exposure, an additional mechanism to protect against BPH.

The role of inflammation has been well-documented in BPH development in several studies (Robert et al., 2009; Krušlin et al., 2017). Inflammatory mediators and the presence and degree of inflammation are recognized to be key-players in prostate enlargement and hyperplasia (Krušlin et al., 2017; Bostanci et al., 2013). Thus, halting inflammation is a therapeutic target for BPH. The origin of inflammation in BPH is likely to be multifactorial; however, immune cell accumulation in the prostate, particularly T lymphocytes and macrophages, create an atypical inflammatory microenvironment. These cells, along with epithelial and stromal cells, trigger inflammatory pathways and cause a wide variety of cytokines that participate in pathology for BPH (Krušlin et al., 2017). The infiltration of macrophages was increased in human and mouse BPH tissues (Wang et al., 2012). Macrophages in particular produce several cytokines, such as CCL-5, CCL-2, IL-1β, and IL-6 (Krušlin et al., 2017). In addition, in a co-culture Transwell system, increased migration of macrophages promoted prostate stromal cell proliferation via AR/CCL3 (Wang et al., 2012). Furthermore, recruitment of macrophages was positively correlated to prostate cancer progression (Karan and Dubey, 2016). Wang et al., (2004) reported that macrophages induce cyclooxygenase 2 (COX-2) expressions in prostatic epithelium in BPH associated with chronic inflammation.

Presently, we demonstrated that ICA prevented testosterone-increased macrophage accumulation in prostate tissues, which eventually can inhibit stromal cell proliferation and BPH-associated inflammation. Macrophages also express iNOS that produces NO, causing an exacerbation of lipid peroxidation-induced BPH. Therefore, our data suggest an additional mechanism by which ICA attenuates BPH through inhibiting macrophages' accumulation.

5. CONCLUSION

The current study provides new evidences for the use of ICA as a potential anti-BPH plant resource. The results confirmed that ICA had a protective effect against testosterone-induced BPH in rats. ICA successfully ameliorated hyperplasia, oxidative stress and macrophage infiltration in BPH rats. The exact mechanism by which ICA exerts its anti-BPH effect will be investigated in the future.

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Authors' Contributions

All authors contributed to the research and/or preparation of the manuscript. Thikryat Neamatallah (TN) and Basma G. Eid (BE) designed the study and participated in the lab work. BE performed the statistical analyses. TN wrote the first draft of the manuscript while BE proofread it. All authors read and approved the final manuscript.

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This study has not received any external funding.

Conflicts of Interest

The author declares that there are no conflicts of interests

Ethics Approval

The protocol of the study was approved by The Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (reference number 1438-107).

Data and materials availability

All data associated with this study are present in the paper.

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